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Patent- og Varemærkestyrelsen
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Susanne Morsing

AMYLASE

- 8 JAN. 2004

PVS

FIELD OF THE INVENTION

The present invention relates to a polypeptide having amylase activity, to DNA encoding it and to its use in the preparation of dough and dough-based products.

5 BACKGROUND OF THE INVENTION

Pdb file 6taa (available at www.rcsb.org) describes a fungal amylase (Taka amylase) from *Aspergillus oryzae*.

SUMMARY OF THE INVENTION

The inventors have identified an amylase from a fungal strain of *Chaetomium sp.* and
10 found that the amylase can increase the shelf life of baked products. More specifically, the amylase in combination with a maltogenic amylase further improves the softness of bread crumb without having detrimental effects on elasticity.

Accordingly, the invention provides a polypeptide having amylase activity. It may be a polypeptide encoded by the amylase-encoding part of the DNA sequence inserted into a plas-
15 mid present in *E. coli* DSM 16113 or having an amino acid sequence as shown in positions 1-566 of SEQ ID NO 2, or it may be at least 70 % identical to one of these. The polypeptide may also be encoded by a nucleic acid sequence which hybridizes at 55°C with the complementary strand of nucleotides 146-1843 of SEQ ID NO: 1

The invention also provides a polypeptide having an amino acid sequence which can
20 be obtained from the mature polypeptide of SEQ ID NO: 2 by substitution, deletion, and/or insertion of one or more amino acids and a polynucleotide having a sequence that can be derived from SEQ ID NO: 1 by substitution, deletion, and/or insertion of one or more nucleotides.

The invention also provides a polynucleotide encoding the amylase, an expression vector comprising the polynucleotide, a transformed host cell comprising the vector, as well as
25 a method of producing the amylase by cultivating the transformant. The invention further provides a dough composition comprising the amylase, a method of preparing a dough-based product by leavening and heating the dough, e.g. by baking.

DETAILED DESCRIPTION OF THE INVENTION

Genomic DNA source

30 The source organism of the amylase of the invention is a fungal strain isolated from soil samples collected from Hainan Province, China, in 2002. The strain was classified as be-

longing to Fungi, *Ascomycota*, *Sordariomycetidae*, *Sordariales*, *Chaetomiaceae*, *Chaetomium* sp.

The inventors have cloned the gene encoding the polypeptide of the invention from the source organism into a strain of *E. coli* and deposited it under the terms of the Budapest Treaty on 16 December 2003 as DSM 16113 with the DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig DE.

Recombinant expression vector

The expression vector of the invention typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a selectable marker, a transcription terminator, a repressor gene or various activator genes. The vector may be an autonomously replicating vector, or it may be integrated into the host cell genome.

Production by cultivation of transformant

The polypeptide of the invention may be produced by transforming a suitable host cell with a DNA sequence encoding the lipolytic enzyme, cultivating the transformed organism under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

The host organism may particularly be a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, e.g. a strain of *Aspergillus*, *Fusarium*, *Trichoderma* or *Saccharomyces*, particularly *A. niger*, *A. oryzae*, *F. graminearum* or *S. cerevisiae*.

Hybridization

Suitable conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involve presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (standard saline citrate) for 10 min, and prehybridization of the filter in a solution of 5 x SSC (Sambrook et al. 1989), 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ³²P-dCTP-labeled (specific activity > 1 x 10⁹ cpm/µg) probe for 12 hours at approx 45°C. The filter is then washed two times for 30 minutes in 2 x SSC, 0.5 % SDS at a temperature of at least 55°C, more particularly at least 60°C, more particularly at least 65°C, even more particularly at least 70°C, especially at least 75°C. Molecules to which the oligonucleotide probe hybridizes under these conditions may be detected using a x-ray film.

Alignment and identity

The polypeptide and polynucleotide of the invention may have identities to the disclosed sequences of at least 80 %, particularly at least 85 % or at least 90 %, e.g. at least 95 %.

- 5 For purposes of the present invention, alignments of sequences and calculation of identity scores may be done using a Needleman-Wunsch alignment (i.e. global alignment), useful for both protein and DNA alignments. The default scoring matrices BLOSUM50 and the identity matrix are used for protein and DNA alignments respectively. The penalty for the first residue in a gap is -12 for proteins and -16 for DNA, while the penalty for additional residues in
10 a gap is -2 for proteins and -4 for DNA. Alignment is from the FASTA package version v20u6 (W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448, and W. R. Pearson (1990) "Rapid and Sensitive Sequence Comparison with FASTP and FASTA", Methods in Enzymology, 183:63-98).

Dough

- 15 The dough of the invention generally comprises flour, particularly wheat flour. The dough may be fresh, frozen or par-baked. It may be a laminated dough.

The dough may also comprise other conventional dough ingredients, e.g., proteins, such as milk powder and gluten; eggs (either whole eggs, egg yolks or egg whites); an oxidant such as ascorbic acid, potassium bromate, potassium iodate, azodicarbonamide (ADA) or
20 ammonium persulfate; an amino acid such as L-cysteine; a sugar; a salt such as sodium chloride, calcium acetate, sodium sulfate or calcium sulfate. The dough may comprise fat (triglyceride) such as granulated fat or shortening.

Additional enzyme

- Optionally, one or more additional enzymes may be added to the dough together with
25 the amylase of the invention. The additional enzyme may be a second amylase, a lipolytic enzyme (e.g. as described in WO 9953769) or a xylanase. The second amylase may be an exo-acting maltogenic alpha-amylase, e.g. as described in WO 9104669 or WO 9943794; an example is Novamyl® (product of Novozymes A/S)

Dough-based product

- 30 The invention provides a method for preparing a dough-based product by leavening the dough and heating it, e.g. by baking or steaming. The dough may be leavened e.g. by adding chemical leavening agents or yeast, usually *Saccharomyces cerevisiae* (baker's yeast). The product may be of a soft or a crisp character, either of a white, light or dark type. Exam-

ples are steamed or baked bread (in particular white, whole-meal or rye bread), typically in the form of loaves or rolls.

Amylase Units (AmU)

Amylase activity was assayed by incubating with Phadebas amylase test tablets (product of Amersham Pharmacia) suspended in 50 mM sodium acetate + 1 mM CaCl_2 at pH 5.7 and determining OD at 650 nm. The Amylase Unit (AmU) was defined by taking the activity of the commercial product BAN 480L (product of Novozymes A/S) as 480 AmU.

EXAMPLES

Materials and methods

- 10 RNeasy Mini Kit (Qiagen, Cat. #74904).
- Taq DNA polymerase (Promega, Cat. # M166A)
- pGEM-T Vector System I (Promega, Cat. # A3600)
- Wizard Plus Minipreps DNA Purification System (Promega, Cat. # A7510)
- 5' Rapid Amplification of cDNA End System (Life Technologies, 5'RACE, Cat # 18374-
- 15 041),
- 3' Rapid Amplification of cDNA End System (Life Technologies , 3' RACE, Cat. # 1085805)
- ElectroMAX DH10B Cells (Life Technologies, Cat. # 18290-015)

Example 1: Cultivation of fungal strain for cDNA preparation

20 The fungal strain *Chaetomium sp.* was grown on YG agar plate (4.5 cm diam) for 5 days under 37°C in the darkness and used for inoculating shake flask. The plates with fully grown cultures were stored at 4°C before use.

To obtain the mycelium for cDNA library construction, 4-6 agar plugs with fully grown fungal cultures on the YG agar plates were used to inoculate one shake flask with FG-4 (50 ml in 500 ml Erlenmeyer flask with 2 baffles: 30 g Soymeal, 15 g Maltose, 5 g Peptone, 1000 ml H₂O, 1% starch, 1 g olive oil (2 drops / flask), Autoclave at 121 °C for 30 min) and grown under 37°C, 160 rpm for 24 hours. The mycelium was harvested by centrifugation of the culture broth at 8000 rpm and 4°C for 30 minutes. Then mycelium was transferred into a clean plastic bag following by immediately freezing in liquid nitrogen and stored at -80°C before total RNA was isolated.

30

Probe designing:

Degenerate primers were designed based on alignment of already known amylase gene sequences. amyD1 (SEQ ID NO: 3) and amyD2R (SEQ ID NO: 4).

The N-terminal amino acid sequence of the purified amylase AM835F was determined as shown in SEQ ID NO. 13. This was used to design four degenerate primers: AM835n-s1 (SEQ ID NO: 5), AM835n-s2a (SEQ ID NO: 6), AM835n-s2b (SEQ ID NO: 7), AM835n-s2c (SEQ ID NO: 8).

Extraction of total RNA:

Total RNA was isolated from the frozen mycelium of a strain of *Chaetomium* sp. by using RNeasy Mini Kit according to the manufacturer's instructions.

Gene cloning:

cDNA was synthesized using 3' RACE kit. The primary PCR was performed by using N-terminal based degenerate primers (primer AM835n-s2 is a mixture of AM835n-s2a, b and c) with AUAP provided by the 3'RACE kit:

10X PCR buffer	5 micro-l
25mM MgCl ₂	3 micro-l
10mM dNTP	1 micro-l
100 micro-M AM835n-s2a	1 micro-l
100 micro-M AM835n-s2b	1 micro-l
100 micro-M AM835n-s2c	1 micro-l
AUAP	1 micro-l
cDNA	2 micro-l
Taq DNA polymerase (5u/ micro-l)	1 micro-l
H ₂ O	34 micro-l

The PCR program was: 94°C for 3 min; 30 cycles of 94 °C for 30s, 50°C for 30s, 72°C for 1.5 min; final extension at 72°C for 10 min.

There was no specific amplification seen when the PCR product was visualized under UV but this product was used for second PCR with degenerate primers designed based on amylase homology. The 2nd PCR was performed by using amylase probes (amyD1 and amyD2R) and using the primary PCR as template:

10X PCR buffer	5 micro-l
25mM MgCl ₂	3 micro-l
10mM dNTP	1 micro-l
100 micro-M amyD1	1 micro-l
100 micro-M amy D2R	1 micro-l
1 st PCR	1 micro-l

Taq DNA polymerase (5u/ micro-l)	1 micro-l
H ₂ O	37 micro-l

The PCR program was: 94°C for 3min; 30 cycles of 94 °C for 30s, 50°C for 30s, 72°C for 1min; final extension at 72°C for 10min. A specific band was amplified at ~700bps and this was confirmed to be amylase by sequencing.

Based on the above obtained partial sequence, new primers were designed for 5' and 3' end cloning. For 5' end cloning, after cDNA was synthesized initiated with amy835as1 (SEQ ID NO: 9) by 5'RACE kit, PCR was performed with primer pairs amy835as1 and AAP (provided by the kit). Then nested PCR was performed with primer pair amy835as3 (SEQ ID NO: 14) and AUAP by using primary PCR (amy835as1-AAP) as template. A fragment of ~600bp was obtained and confirmed by sequencing. For 3' end cloning, PCR was performed by using primer pair of amy835f1 (SEQ ID NO: 15) and AUAP and cDNA as template. The nested PCR was performed by using primer pair amy835f2 (SEQ ID NO: 10) with AUAP and 1st PCR as template. A fragment at ~600 bps was amplified and again confirmed by sequencing.

Then based on the cloned 5' and 3' end sequences, the 5' and 3' end primers for full length cloning was designed and used for full length cloning of the amylase AM835. By using cDNA synthesized by 3' RACE kit as template and probes amy835s00 (SEQ ID NO: 11) and amy835as01 (SEQ ID NO: 12) as primers:

10X PCR buffer	5 micro-l
25mM MgCl ₂	3 micro-l
10mM dNTP	1 micro-l
10 micro-M amy835s00	1 micro-l
10 micro-M amy835as01	1 micro-l
cDNA	2 micro-l
Taq DNA polymerase (5 u/micro-l)	1 micro-l
H ₂ O	36 micro-l

PCR program was: 94°C for 3 min; 30 cycles of 94 °C for 30s, 50°C for 30s, 72°C for 1.5 min; final extension at 72°C for 10 min.

A specific fragment of ~2.0 kb was PCR-amplified. The fragment was cloned into pGEM-T vector (Promega) which has a 3'-T overhang and transformed into *E.coli* DH10B (ElectroMAX DH10B Cells, available from Life Technologies, Cat. # 18290-015) and further sequenced.

Example 2: Production of amylase

YG and FG-4 media were prepared as follows.

YG: Yeast-glucose agar

5.0 g Difco powdered yeast extract;

10.0 g glucose

20.0 g agar;

1000 ml tap water

Autoclave at 121 °C for 15-20 min.

5 FG-4 Media 50 ml / flask:

30 g Soymeal,

15 g Maltose

5 g Peptone,

1000 ml H₂O

1 g olive oil (2 drops / flask)

50 ml in 500 ml Erlenmeyer flask with 2 baffles. Autoclave at 121 °C for 30 min

- 10 A strain of the thermophilic fungus *Chaetomium sp.* was grown on YG agar plate (4.5 cm diam) for 3 days under 37 °C in the darkness and used for inoculating shake flask. The plates with fully grown cultures were stored at 4 °C before use.

For enzyme production, 4-6 agar plugs with fully grown fungal cultures on the above plates were used to inoculate one shake flask with FG-4 and grown under 37 °C, 160 rpm for 15 72 hours, then harvested by centrifuged the culture broth at 8000 rpm and 4 °C for 30 minutes. The supernatant was collected and used for enzyme purification.

1000 ml supernatant was precipitated with ammonium sulfate (80% saturation) and redissolved in 100 ml 25mM Tris-HCl buffer, pH7.0, then dialyzed against the same buffer and filtered through a 0.45 mm filter, the final volume was 200 ml. The solution was applied to a 35 20 ml Source 15Q column (Pharmacia) equilibrated in 25 mM Tris-HCl buffer, pH7.0, and the proteins was eluted with a linear NaCl gradient (0 –0.3M). Fractions from the column were analyzed for amylase activity on AZCL-amylase at pH 5.5. Fractions with amylase activity were pooled. Then the pooled solution was ultrafiltrated, the concentrated solution was applied to a 180ml Superdex75 column equilibrated with 25 mM Tris-HCl, pH7.0, the proteins was eluted 25 with the same buffer. Amylase containing fractions were analyzed by SDS-PAGE and pure fractions were pooled.

The purified amylase was used for characterization in the following example.

Example 3: Expression of an amylase from *Chaetomium sp.* in *Aspergillus oryzae*

The DNA sequence of the *Chaetomium* amylase (SEQ ID NO.: 1) was used to design 30 primers for PCR amplification of the amylase encoding-gene from the clone described in Example 1, with appropriate restriction sites added to the primer ends to facilitate sub-cloning of the PCR product (primers AM835.1 and AM835.2, SEQ ID NO: 16 and 17). PCR amplification was performed using AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions and using an annealing temperature of 55 °C for 35 the first 5 cycles and 65 °C for an additional 25 cycles and an extension time of 2 minutes

The PCR fragment was restricted with *Bam*HI and *Xho*I and cloned into the *Aspergillus* expression vector pMStr57 using standard techniques. The expression vector pMStr57 contains the same elements as pCaHj483 (WO 98/00529), with minor modifications made to the *Aspergillus* NA2 promoter as described for the vector pMT2188 in WO 01/12794, and has 5 sequences for selection and propagation in *E. coli*, and selection and expression in *Aspergillus*. Specifically, selection in *Aspergillus* is facilitated by the *amdS* gene of *Aspergillus nidulans*, which allows the use of acetamide as a sole nitrogen source. Expression in *Aspergillus* is mediated by a modified neutral amylase II (NA2) promoter from *Aspergillus niger* which is fused to the 5' leader sequence of the triose phosphate isomerase (*tpi*) encoding-gene from 10 *Aspergillus nidulans*, and the terminator from the amyloglucosidase-encoding gene from *Aspergillus niger*. The amylase-encoding gene of the resulting *Aspergillus* expression construct, pMStr91, was sequenced and the sequence agreed completely with that determined previously.

The *Aspergillus oryzae* strain BECh2 (WO 00/39322) was transformed with pMStr91 15 using standard techniques (Christensen, T et al., (1988), Biotechnology 6, 1419-1422). Transformants were cultured in YP+2%G medium shaken at 250 RPM at 30°C and expression of amylase was monitored by SDS-PAGE.

Medium YP+2%G

10g yeast extract
20 20g peptone
water to 1L
autoclave at 121°C, 20 minutes
add 100ml 20% sterile glucose solution

Example 4: Characterization of amylase

25 The molecular weight of the amylase prepared in a previous example was found to be around 66 kDa as seen on SDS-PAGE. The isoelectric point (pI) was found to be around pH 3.5, as determined by isoelectric focusing (IEF).

pH and temperature profiles were determined with AZCL-amylose (product of Megazyme) as substrate. At 50°C, the amylase was found to be active at pH 4-10 with an optimum around pH 5-7. At pH 5.5, the amylase was found to be active at 20-70°C with an optimum around 60°C. Thus, the *Chaetomium* amylase has a wider pH range and a higher temperature optimum than the fungal amylase from *Aspergillus oryzae*.

Stability of the amylase was determined by incubation at pH 5-7 and 60-80°C for 5- 25 minutes. The results showed more than 90 % residual activity after 20 minutes at pH 6-7 and 35 60°C. At pH 5.0 and 60°C, the amylase was nearly completely inactivated in 15 minutes. At

70°C, the amylase was nearly completely inactivated at in 5-10 minutes at pH 6-7. It was found that at all conditions the *Chaetomium* amylase is more stable than the fungal amylase from *Aspergillus oryzae*

The amylase showed no activity on the following substrates at pH 7.0: AZCL-galactomannan, AZCL-beta-glucan, AZCL-dextran, AZCL-xyloglucan, AZCL-potato galactan, AZCL-arabinan, AZCL-pullulan, AZCL-xylan, AZCL-he-cellulose and AZCL-casein

Example 5: Effect of amylase on freshness of bread

Bread were baked according to the sponge & dough method.

Recipes

<u>Sponge</u>	<u>% on flour basis</u>
Soya oil	2,5
Sodium stearoyl lactylate (SSL)	0,38
Yeast	5
Wheat flour	60
Water	62

<u>Dough</u>	<u>% on flour basis</u>
Ascorbic acid	optimized for each flour
ADA	20 ppm
Salt	2
Syrup	7 (dry substance)
Water	optimized for each flour
Wheat flour	40
Calcium propionate	0 25
Enzymes	as indicated below

10 Sponge

Scaling of ingredients, addition of yeast, water, flour, SSL and oil into mixer bowl

Mixing 90 rpm for 1 minutes, 150 rpm for 4 minutes

The sponge was weighted, the temperature was measured and the sponge was placed in a bowl ~ fermentation 3 hours at 27 C, 86 % RH

15 Dough

Addition of ingredients and the sponge into the mixer bowl. The sponge and ingredients were mixed together 90 rpm for 9 minutes

The temperature was measured, dough characteristics were evaluated, the dough was scaled into smaller pieces of 435 g each.

The dough rests on the table for 10 minutes

Doughs were sheeted and molded.

5 Fermentation for 55 minutes at 42°C and 86% RH.

Bread were baked at 200°C for 22 minutes

Enzymes were dosed at 400 MANU/kg of Novamyl together with 0, 5 or 20 AmU/kg of the amylase of SEQ ID NO: 2 (prepared as in Example 1).

Bread were stored at room temperature until analysis.

10 Texture and water migration by NMR were measured on day 7, 14 and 21. A small sensory evaluation of softness and moistness was performed on day 21

Results

Firmness of the loaves was measured as described in WO 9953769 The results were as follows:

Novamyl dosage MANU/kg	Amylase of in- vention AmU/kg	Firmness after 7 days g	Firmness after 14 days g	Firmness after 21 days g
400	0	593	869	1103
400	5	505	814	1000
400	20	480	789	939

15

Elasticity of the loaves was measured as described in US 6162628. The results were as follows:

Novamyl dosage MANU/kg	Amylase of in- vention AmU/kg	Elasticity after 7 days %	Elasticity after 14 days %	Elasticity after 21 days %
400	0	50.7	46.5	45.2
400	5	50.1	46.7	44.7
400	20	50.7	47.2	46.0

20 The data show that the amylase of the invention has a significant effect on firmness in combination with Novamyl, furthermore the elasticity seems to be comparable to or even better than that of Novamyl after 21 days of storage.

The mobility of free water was determined as described by P. L. Chen, Z. Long, R. Ruan and T. P. Labuza, Nuclear Magnetic Resonance Studies of water Mobility in Bread during Storage Lebensmittel Wissenschaft und Technologie 30, 178-183 (1997). The results were
25 as follows:

Novamyl dosage MANU/kg	Amylase of In- vention AmU/kg	Free water after 7 days Micro-sec	Free water after 14 days Micro-sec	Free water after 21 days Micro-sec
400	0	7498	6921	6198
400	5	7780	6856	6424
400	20	7945	7004	6618

The data show that the amylase of the invention increases the amount of free water. The amount of free water has been described in literature to correlate to moistness of bread crumb.

- 5 The ranking from the small sensory evaluation of softness and moistness on day 21 showed the following ranking (MANU/kg of Novamyl + AmU/kg of amylase of invention):

Moistest: 400 MANU + 20 AmU

Second: 400 MANU + 5 AmU

Lowest (least moist): 400 MANU

CLAIMS

1. A polypeptide having amylase activity selected from the group consisting of:
 - a) a polypeptide encoded by the amylase-encoding part of the DNA sequence inserted into a plasmid present in *E. coli* DSM 16113
 - 5 b) a polypeptide having an amino acid sequence as shown in positions 1-566 of SEQ ID NO 2,
 - c) a polypeptide which has at least 70 % identity to the polypeptide defined in (a) or (b),
 - d) a polypeptide which is encoded by a nucleic acid sequence which hybridizes at 55
 - 10 °C with the complementary strand of nucleotides 146-1843 of SEQ ID NO: 1.
2. A polynucleotide comprising a sequence selected from the group consisting of:
 - a) the amylase-encoding sequence inserted into a plasmid present in *E. coli* DSM 16113;
 - b) nucleotides 146-1843 of SEQ ID NO: 1;
 - 15 c) a polynucleotide encoding amino acids 1-566 of SEQ ID NO 2;
 - d) a polynucleotide which encodes a polypeptide having amylase activity and has at least 70 % identity to the polynucleotide of a), b) or c),
 - e) a nucleic acid sequence which hybridizes at 55°C with the complementary strand of the polynucleotide of a), b) or c),
 - 20 f) the complementary strand of the polynucleotide of a), b), c), d) or e).
3. A vector comprising the polynucleotide of claim 2 operably linked to one or more control sequences that direct the production of the polypeptide in a suitable host.
4. A transformed host cell comprising the vector of claim 3.
5. A method for producing an amylase, which comprises
 - 25 a) cultivating the host cell of claim 4 under conditions appropriate for expression of amylase, and
 - b) recovering the amylase.
6. A dough composition which comprises flour and the polypeptide of claim 1.
7. A process for preparing a dough-based product, comprising adding the polypeptide of
- 30 claim 1 to a dough, leavening, and heating the dough.

8. The process of claim 7 which further comprises adding an exo-acting maltogenic alpha-amylase to the dough

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10583-000.ST25
SEQUENCE LISTING

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ttc Phe	tcc Ser 215	ggc Gly	gac Asp	acc Thr	tcc Ser	tac Tyr 220	gtg Val	gcc Ala	ggc Gly	tat Tyr	caa Gln 225	tgc Ser	gtg Val	atg Met	cgc Pro	832
ggc Gly 230	ttg Leu	ctc Leu	aac Asn	tat Tyr	ccc Pro 235	atc Ile	tac Tyr	tat Tyr	ccg Pro	ctc Leu 240	atc Ile	cgc Arg	gtc Val	ttc Phe	gcg Ala 245	880
cag Gln	ggt Gly	gcg Ala	tcc Ser	ttc Phe 250	acc Thr	gat Asp	ctc Leu	gtc Val	aac Asn 255	aac Asn	cac His	gat Asp	acc Thr	gtc Val 260	ggc Gly	928
tgc Ser	acc Thr	ttc Phe 265	tcc Ser	gac Asp	ccg Pro	acg Thr	ctg Leu	ctg Leu 270	ggt Gly	aac Asn	ttt Phe	atc Ile	gac Asp 275	aac Asn	cac His	976
gac Asp	aac Asn	cca Pro 280	cgt Arg	ttc Phe	ctg Leu	agc Ser	tac Tyr 285	acc Thr	agc Ser	gac Asp	cac His	gcc Ala 290	ctc Leu	ctc Leu	aag Lys	1024
aac Asn 295	gct Ala	ctg Leu	gcc Ala	tac Tyr	gtc Val	atc Ile 300	ctg Leu	gcc Ala	aga Arg	ggc Gly	atc Ile 305	ccc Pro	atc Ile	gtc Val	tac Tyr	1072
tac Tyr 310	ggc Gly	acc Thr	gag Glu	caa Gln	ggc Gly 315	tac Tyr	tgc Ser	ggt Gly	tgc Ser	tcc Ser 320	gac Asp	ccg Pro	gcg Ala	aac Asn	cgc Arg 325	1120
gag Glu	gat Asp	ctc Leu	tgg Trp	cgt Arg 330	agc Ser	gga Gly	tac Tyr	agc Ser	act Thr 335	acg Thr	gga Gly	gac Asp	atc Ile	tac Tyr 340	acc Thr	1168
acc Thr	atc Ile	gcc Ala 345	gcg Ala	ctc Leu	tcc Ser	gcc Ala	gcg Ala 350	cgc Arg	acc Thr	gcg Ala	gcc Ala	ggt Gly 355	ggc Gly 355	ctc Leu	gcc Ala	1216
ggt Gly	aac Asn 360	gac His	cac His	gtc Val	cac His	ctg Leu 365	tac Tyr	acg Thr	acc Thr	gac Asp	aac Asn	gcg Ala 370	tac Tyr	gcc Ala	tgg Trp	1264
tcc Ser 375	cgc Arg	gcg Ala	agc Ser	ggc Gly	aag Lys	ctc Leu 380	atc Ile	gtc Val	gtc Val	acg Thr	tcc Ser 385	aac Asn	cgc Arg	ggc Gly	agc Ser	1312

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tcc gac agc agc acc atc tgc ttc agc acc cag cag gcc agc ggc acc Ser Asp Ser Ser Thr Ile Cys Phe Ser Thr Gln Gln Ala Ser Gly Thr 390 395 400 405	1360
acc tgg acc agc acg atc acc ggc aac tcg tac acc gcc gac agc aac Thr Trp Thr Ser Thr Ile Thr Gly Asn Ser Tyr Thr Ala Asp Ser Asn 410 415 420	1408
ggc cag atc tgc gtg cag ctg tcc agc ggc gga ccc gag gcg ctc gtc Gly Gln Ile Cys Val Gln Leu Ser Gly Gly Pro Glu Ala Leu Val 425 430 435	1456
gtc tcc acc gcg acc ggc acc gcc acc gcg acg act ctg tcc acg acc Val Ser Thr Ala Thr Gly Thr Ala Thr Thr Leu Ser Thr Thr 440 445 450	1504
acc aag acg tcc acc tcg acc gcc tcc tgc gcc gcc acc gtc gcc gtc Thr Lys Thr Ser Thr Ser Ala Ser Cys Ala Thr Val Ala Val 455 460 465	1552
acc ttc aac gag ctc gtc acc acg aac tac ggc gac acc atc cgc ctg Thr Phe Asn Glu Leu Val Thr Thr Asn Tyr Gly Asp Thr Ile Arg Leu 470 475 480 485	1600
acg ggc tcc atc tcc cag ctg agc agc tgg agc gca acc tcc ggc ctg Thr Gly Ser Ile Ser Gln Leu Ser Ser Trp Ser Ala Thr Ser Gly Leu 490 495 500	1648
gcc ctg agc gcg tcc gcg tac acg tcc agc aac ccg ctc tgg agc gtg Ala Leu Ser Ala Ser Ala Tyr Thr Ser Ser Asn Pro Leu Trp Ser Val 505 510 515	1696
acg gtc agc ctg ccg gcc ggc acg tcg ttc gag tac aag ttc gtc cgc Thr Val Ser Leu Pro Ala Gly Thr Ser Phe Glu Tyr Lys Phe Val Arg 520 525 530	1744
atc acg agc gac ggc acc gtg acc tgg gaa tcg gac ccg aac cgc agc Ile Thr Ser Asp Gly Thr Val Thr Trp Glu Ser Asp Pro Asn Arg Ser 535 540 545	1792
tac acc gtc ccg acg tgc gcg agc acc gcg acg atc agc aat acc tgg Tyr Thr Val Pro Thr Cys Ala Ser Thr Ala Thr Ile Ser Asn Thr Trp 550 555 560 565	1840
cgg tgagctctgg acgtgttgta catataggag gccgttgaga ggccggggcg Arg	1893
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Gln Leu Leu Ala Ala Ser Asn Ser Asp Trp Arg Ser Arg Asn Ile Tyr
 -1 1 5 10

Phe Ala Leu Thr Asp Arg Val Ala Asn Pro Ser Thr Thr Thr Ala Cys

15

20

25

Ser Asp Leu Ser Asn Tyr Cys Gly Gly Thr Trp Ser Gly Leu Ser Ser
 30 35 40
 Lys Leu Asp Tyr Ile Gln Gly Met Gly Phe Asp Ser Ile Trp Ile Thr
 45 50 55 60
 Pro Val Val Glu Asn Cys Asp Gly Gly Tyr His Gly Tyr Trp Ala Lys
 65 70 75
 Ala Leu Tyr Asn Val Asn Thr Asn Tyr Gly Ser Ala Asp Asp Leu Lys
 80 85 90
 Asn Phe Val Ala Ala Ala His Ala Lys Gly Met Tyr Val Met Val Asp
 95 100 105
 Val Val Ala Asn His Met Gly Ser Cys Gly Ile Ala Asn Leu Ser Pro
 110 115 120
 Pro Pro Leu Asn Glu Gln Ser Ser Tyr His Thr Gln Cys Asp Ile Asp
 125 130 135 140
 Tyr Ser Ser Gln Ser Ser Ile Glu Thr Cys Trp Ile Ser Gly Leu Pro
 145 150 155
 Asp Leu Asp Thr Thr Asp Ser Thr Ile Arg Ser Leu Phe Gln Thr Trp
 160 165 170
 Val His Gly Leu Val Ser Asn Tyr Ser Phe Asp Gly Leu Arg Val Asp
 175 180 185
 Thr Val Lys His Val Glu Lys Asp Tyr Trp Pro Gly Phe Val Ser Ala
 190 195 200
 Ala Gly Thr Tyr Ala Ile Gly Glu Val Phe Ser Gly Asp Thr Ser Tyr
 205 210 215 220
 Val Ala Gly Tyr Gln Ser Val Met Pro Gly Leu Leu Asn Tyr Pro Ile
 225 230 235
 Tyr Tyr Pro Leu Ile Arg Val Phe Ala Gln Gly Ala Ser Phe Thr Asp
 240 245 250
 Leu Val Asn Asn His Asp Thr Val Gly Ser Thr Phe Ser Asp Pro Thr
 255 260 265
 Leu Leu Gly Asn Phe Ile Asp Asn His Asp Asn Pro Arg Phe Leu Ser
 270 275 280
 Tyr Thr Ser Asp His Ala Leu Leu Lys Asn Ala Leu Ala Tyr Val Ile

285 290 300
Leu Ala Arg Gly Ile₃₀₅ Pro Ile Val Tyr Tyr₃₁₀ Gly Thr Glu Gln Gly₃₁₅ Tyr
Ser Gly Ser Ser₃₂₀ Asp Pro Ala Asn Arg₃₂₅ Glu Asp Leu Trp Arg₃₃₀ Ser Gly
Tyr Ser Thr₃₃₅ Thr Gly Asp Ile Tyr₃₄₀ Thr Thr Ile Ala Ala₃₄₅ Leu Ser Ala
Ala Arg Thr Ala Ala Gly Gly₃₅₅ Leu Ala Gly Asn Asp₃₆₀ His Val His Leu
Tyr Thr Thr Asp Asn Ala₃₇₀ Tyr Ala Trp Ser Arg₃₇₅ Ala Ser Gly Lys Leu₃₈₀
Ile Val Val Thr Ser₃₈₅ Asn Arg Gly Ser Ser₃₉₀ Asp Ser Ser Thr Ile₃₉₅ Cys
Phe Ser Thr Gln₄₀₀ Gln Ala Ser Gly Thr₄₀₅ Thr Trp Thr Ser Thr₄₁₀ Ile Thr
Gly Asn Ser₄₁₅ Tyr Thr Ala Asp Ser₄₂₀ Asn Gly Gln Ile Cys₄₂₅ Val Gln Leu
Ser Ser Gly Gly Pro Glu Ala₄₃₅ Leu Val Val Ser Thr₄₄₀ Ala Thr Gly Thr
Ala Thr Ala Thr Thr Leu₄₅₀ Ser Thr Thr Thr Lys₄₅₅ Thr Ser Thr Ser Thr₄₆₀
Ala Ser Cys Ala Ala₄₆₅ Thr Val Ala Val Thr₄₇₀ Phe Asn Glu Leu Val₄₇₅ Thr
Thr Asn Tyr Gly₄₈₀ Asp Thr Ile Arg Leu₄₈₅ Thr Gly Ser Ile Ser₄₉₀ Gln Leu
Ser Ser Trp₄₉₅ Ser Ala Thr Ser Gly₅₀₀ Leu Ala Leu Ser Ala₅₀₅ Ser Ala Tyr
Thr Ser₅₁₀ Ser Asn Pro Leu Trp₅₁₅ Ser Val Thr Val Ser₅₂₀ Leu Pro Ala Gly
Thr Ser₅₂₅ Phe Glu Tyr Lys₅₃₀ Phe Val Arg Ile Thr₅₃₅ Ser Asp Gly Thr Val₅₄₀
Thr Trp Glu Ser Asp₅₄₅ Pro Asn Arg Ser Tyr₅₅₀ Thr Val Pro Thr Cys₅₅₅ Ala
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560

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565

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<220>
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18

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17

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<220>
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17

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<400> 10
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<210> 11
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<220>
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<210> 13
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<400> 13

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Asp Arg Val Ala
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<220>
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29